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Genetic changes implicated in the etiology of breast cancer have been identified by the detection of loss of heterozygosity at specific loci. Our study utilizes a series of genetic polymorphisms detectable by the polymerase chain reaction (PCR) to look for changing patterns of LOH as breast cancer progresses from intraductal to invasive and then to metastatic disease. The method involves the microdissection each tumor component present from a panel of breast cancer cases, and then to test each component for LOH at loci that are known to show high frequency LOH in breast cancer. The aims were to determine where in progression LOH is first observed and to determine whether LOH correlates with the clinical behavior of the tumor. We have now completed the analysis of LOH at loci on 3p, 9p, 11p, 13q, 16q, 17p, and 17q for a panel of 115 breast cancers. At each locus examined, LOH, when present at all, is usually seen in the intraductal component of the tumor and maintained throughout tumor progression. LOH at 11p was found to show no correlations with the clinical behavior of the tumor. Clinical correlations of LOH at the other loci are currently being analyzed.

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Joseph H. Lichy 3/5/98

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INTRODUCTION

The earliest events in the pathogenesis of breast cancer typically involve the loss of a normal growth regulatory mechanism by a ductal or lobular epithelial cell. Progression of the disease through the stages of intraductal proliferation to invasive carcinoma and then to metastatic disease appears to require additional alterations in growth regulatory pathways. A substantial body of evidence now supports the idea that these alterations in growth regulation result from genetic events such as point mutation, deletion, and gene amplification [1-4]. Our study aims to characterize genetic alterations in breast tumors at the various stages of tumor progression. If metastasis requires additional genetic events beyond those responsible for the intraductal and invasive components of the tumor, one should find genetic alterations in the metastasis that are not present in primary tumor. Alternatively, there may be certain genetic lesions which occur early in tumor development that can predispose a tumor to metastasize without the acquisition of additional genetic defects. The identification of such a lesion would provide an important prognostic indicator, because it would provide a means for predicting the likelihood of the development of metastatic disease in tumors identified at an early stage. The characterization of genetic changes present in individual tumor components thus offers the possibility of identifying new prognostic indicators as well as helping to elucidate the significance of genetic events to tumor progression.

The type of genetic analysis performed in our study is the amplification of polymorphic loci by the polymerase chain reaction (PCR) [5]. This technique permits the detection of loss of heterozygosity (LOH) in tumor specimens relative to normal tissue from the same patient. LOH at specific loci has been observed frequently in breast cancer. High frequency of LOH for a specific genetic marker is thought to imply the presence of a tumor suppressor gene at that locus [3, 4]. In certain cases (e.g., p53 on 17p, DCC on 18q), the loss of one copy of the tumor suppressor gene (LOH) is found in association with mutation of the remaining copy. In such cases, LOH indicates that both copies of the tumor suppressor gene have become inactivated, resulting in the loss of a normal growth regulatory pathway. The PCR methodology also permits the detection of gene amplification, assuming that amplification involves only one of the two copies of the gene present. In breast cancer, amplification of the HER2/neu oncogene is of particular interest because of potential prognostic implications [2].

The general strategy of our study involves the identification of a group of breast cancer cases from the AFIP archives followed by microdissection of the intraductal, infiltrating, and metastatic components present in each tumor, and analysis of each tumor component for LOH at multiple genetic loci. The results should help address questions such as when during tumor progression specific genetic lesions occur, and whether LOH at any particular locus has value in predicting the course of progression of an individual tumor. In addition, through the analysis of multiple closely linked markers, the boundaries of each region of LOH can be identified. Comparison of multiple cases showing interstitial deletions often demonstrates a narrow region where these deletions overlap one another. The identification of such a region

of overlap suggests the existence of a tumor suppressor gene in the common segment of overlapping LOH.

BODY

Experimental Methods. 115 cases diagnosed as carcinoma of the breast were retrieved from the AFIP archives. These cases were chosen from those submitted to the institute between 1975 and 1982 so that survival data could be generated over at least a 15 year time period from the initial diagnosis. Specimens were analyzed microscopically to identify regions of intraductal, infiltrating, and metastatic carcinoma, which were then isolated by microdissection. If available, a lymph node section was taken as the normal control for each case; otherwise, normal breast tissue was used. Tissue lysates containing PCR amplifiable DNA were prepared by a standard proteinase K digestion technique. This resulted in approximately four hundred and fifty specimens. These lysates were analyzed by PCR for the presence of polymorphic markers on chromosomes 3p, 9p, 11p, 13q, 16q, 17p, and 17q. The PCR primer sequences were obtained from the Genethon database. At least two markers were used for each of these loci. A more detailed study, aimed at narrowing the smallest region of overlap, was carried out for chromosome 11p15. For this study, the entire collection of lysates was analyzed for LOH at ten different polymorphic markers over an approximately 10 megabase region of 11p15. PCR products were labeled with ^{32}P by kinasing one of the primers. Reaction products were separated on a denaturing polyacrylamide gel and identified by autoradiography. A reduction in allele ratio of greater than 50% relative to the normal control was interpreted as loss of heterozygosity (LOH).

Results. We have now completed the generation of the basic body of data which we proposed to produce in our grant application. A detailed study of LOH at 11p15 has been completed and a manuscript describing the results is currently under review. A copy of this manuscript has been included with this report. The data on 11p15 defined a smallest region of overlap between the markers D11S1318 and D11S4046, demonstrated that LOH at this locus usually occurs by the time the tumor has progressed to the stage of intraductal carcinoma, and argued that LOH at this locus has no correlation with the clinical behavior of the tumor.

The data on the other loci examined show a similar pattern to that observed at 11p15, in that LOH is usually present at the intraductal carcinoma stage and maintained throughout subsequent stages of progression. We have not conducted detailed studies of these loci to characterize smallest regions of overlap as was done for 11p15 because such studies of each of these regions have appeared in the literature since we initiated our own work, and we felt that unless we devoted all our efforts to one locus we were unlikely to contribute anything novel by such studies. We have organized the data on LOH during progression for each locus examined into a summary table in preparation for publication. The table of results for 16q has been included as Appendix A as an example. In this table, cases are categorized by the most advanced tumor stage present. For each marker, results are first given as (# with LOH)/(# informative). The results are then divided by tumor stage, showing (# with LOH in specific tumor component)/(# with LOH in any component). We are currently in the process of

analyzing the results for correlation with clinical parameters to determine whether LOH at any of these loci could be a useful prognostic indicator.

Analysis of the body of data generated as described above yielded an observation which we find intriguing, and which has formed the basis of additional studies which are now the focus of our research. These additional studies follow the essential scheme outlined in the grant proposal but make modifications to take into account our own initial results as well as new insights into breast cancer genetics which have appeared in the literature in the past two years. Our own observation is that LOH can be present in one tumor component but absent in a specimen representing a more advanced stage of tumor progression. This implies, for example, that the metastatic tumor present in a lymph node does not necessarily derive from the invasive tumor present in the surgically resected specimen. In each case where such a phenomenon has been observed, our data are consistent with the possibility that both tumor components share a common precursor. The presence of genetically divergent clones in resected breast cancer specimens has been reported in two studies, one focusing on multiple foci of intraductal carcinoma [6] and the other asynchronous metastases [7]. By inferring the existence of a common precursor cell from shared genetic lesions in tumor components that have genetically diverged, it becomes possible to construct an "evolutionary tree" for each tumor analyzed. As a result of another recent study [8], which demonstrated that LOH can be observed in morphologically normal tissue adjacent to carcinoma, it seemed that such evolutionary trees could be extended back to include lesions earlier than intraductal carcinoma, such as benign proliferations and normal ducts and lobules.

Based on these considerations, we elected to extend our study by conducting additional microdissections of cases that had given evidence of genetic divergence, now including foci of normal and premalignant epithelium in addition to the malignant foci which were initially studied. We have carried out such extensive microdissections on six of these cases. The resulting lysates were characterized for LOH at a panel of markers which we knew worked well from our initial studies. The results from each case have been used to infer the degree of clonal relatedness of the different foci dissected from each tumor specimen. This analysis has revealed an unexpected degree of heterogeneity among tumor components presumed to represent successive stages of progression. Our data has also succeeded in reproducing the observation that LOH can be present in normal tissue adjacent to the carcinoma. Examples of the kinds of evolutionary trees that have been constructed are included as Appendix B. In these diagrams, foci of morphologically normal epithelium are labeled "N," and intraductal, invasive, and metastatic tumor components labeled "ID," "INV," and "MET." Where more than one example of a particular component was analyzed, numbers have been added to the labels to identify each focus individually (e.g.,"N1," "N2"). Precursors inferred from the genetic data are indicated by open circles without specific labels. LOH results are presented as a list of alleles lost (minus sign, "-") or showing microsatellite instability ("MSI"). Where different tumor components have lost different alleles at the same marker, the alleles are designated "A" and "B."

Conclusions. Several conclusions can now be stated from our molecular analysis of a large panel of breast cancer cases. At the outset we hypothesized that certain genetic lesions

would characteristically occur at specific stages of tumor progression. This hypothesis was based on reports in the literature which suffered the limitation that the numbers of both cases and markers analyzed were small. Our results argue that this hypothesis is false, that LOH at all of the loci examined occurs most commonly by the time the tumor has progressed to the intraductal carcinoma stage. Our data reveal no preferential order in which LOH occurs: LOH at any of the loci can occur early or late in progression. With respect to LOH at 11p15, our data specifically refute the claims that this genetic lesion is a late event in breast cancer progression, and that it is a useful prognostic indicator, but confirm the localization of the smallest region of overlap reported by others. We have confirmed the observation that LOH can be detected in normal tissue adjacent to the carcinoma. By conducting extensive microdissections of cases showing genetic heterogeneity, we have shown that what we initially interpreted as lesions representing successive stages of progression present in surgically resected specimens often represent divergent pathways of tumor evolution. One important implication of this result is that it is now apparent that one can not assume that metastatic disease that may develop years after resection of a primary tumor will contain the same genetic lesions present in the resected specimen. It will be important to take the genetic heterogeneity of breast cancer into consideration when developing strategies for early detection of recurrent disease that rely on detecting genetic alterations in the tumor.

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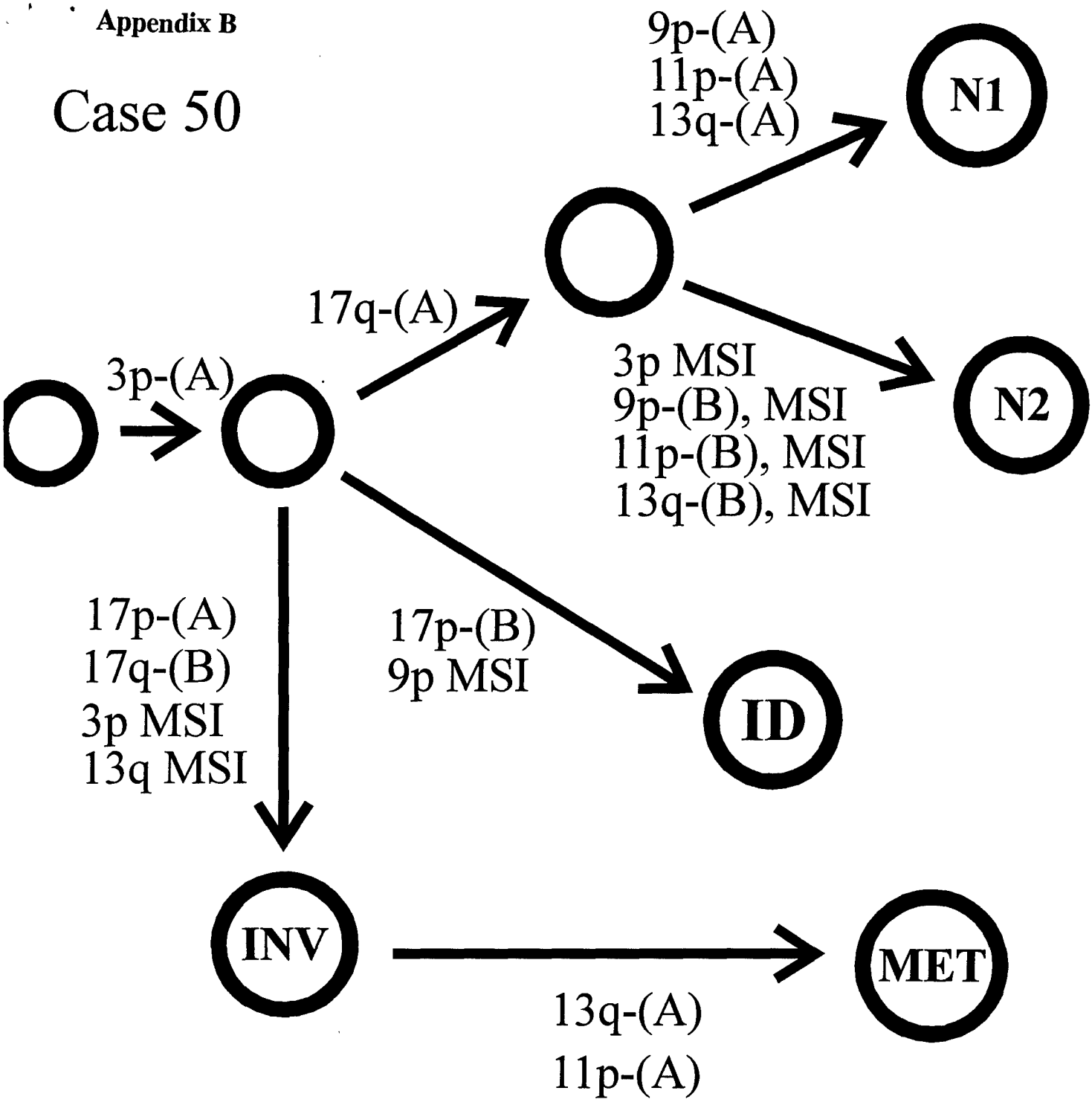
Appendix A

Summary of LOH data during histologic progression for chr 16q

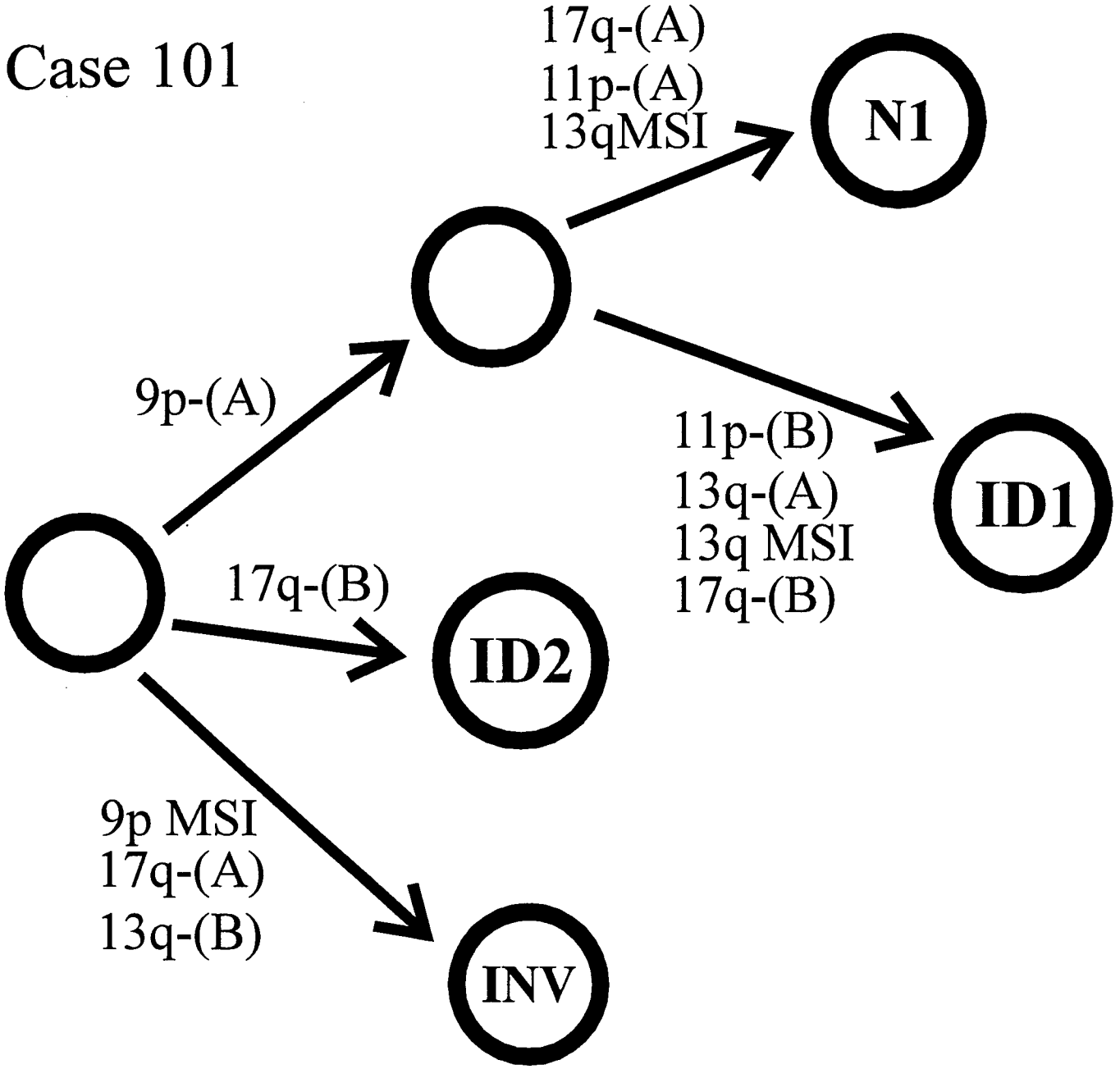
Histologic Diagnosis	No. Marker	No. Cases	LOH at 16q by stage of progression				
			Any Comp.	Intraductal	Invasive	Metastasic	Recurrent
Intraductal	D16S421	8	1/1(100%)	1/1(100%)	NA	NA	NA
	D16S496	8	1/3(34%)	1/1(100%)	NA	NA	NA
carcinoma only	D16S512	8	2/2(100%)	2/2(100%)	NA	NA	NA
Invasive carcinoma	D16S421	58	5/14(36%)	3/4(75%)	4/5(80%)	NA	NA
	D16S496	58	20/30(67%)	9/15(60%)	16/20(80%)	NA	NA
without metastases	D16S512	58	15/27(56%)	10/13(77%)	8/12(67%)	NA	NA
Invasive carcinoma	D16S421	43	11/15(73%)	7/8(88%)	8/11(73%)	7/11(64%)	NA
	D16S496	43	13/19(68%)	10/10(100%)	10/11(91%)	7/12(58%)	NA
with metastases	D16S512	43	14/26(54%)	8/11(73%)	9/13(69%)	10/14(71%)	NA
Invasive, metastases	D16S421	6	1/2(50%)	0/0	0/0	1/1(100%)	1/1(100%)
	D16S496	6	4/4(100%)	0/2	3/4(75%)	4/4(100%)	3/4(75%)
and recurrence	D16S512	6	0	0	0	0	0

Appendix B


Case 50
9p-(A)
11p-(A)
13q-(A)
N1
17q-(A)
3p-(A)
3p MSI
9p-(B), MSI
11p-(B), MSI
13q-(B), MSI
N2
17p-(A)
17q-(B)
3p MSI
13q MSI
17p-(B)
9p MSI
ID
INV
MET
13q-(A)
11p-(A)

Appendix B

Case 101



Loss of Heterozygosity on Chromosome 11p15 During Histologic Progression in Microdissected Ductal Carcinoma of the Breast

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Running head: Chromosome 11p15 LOH in Breast Cancer

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ABSTRACT

Microdissection of histologically identifiable components from formalin fixed paraffin embedded tissue sections allows molecular genetic analyses to be correlated directly with pathologic findings. In this study we have characterized loss of heterozygosity (LOH) at chromosome 11p15 at different stages of progression in microdissected tumor components from 115 ductal carcinomas of the breast. Microdissected foci of intraductal, infiltrating, and metastatic tumor were analyzed to determine the stage of progression at which LOH at 11p15 occurs. LOH was detected in 43 (37%) of 115 cases. Foci of intraductal carcinoma could be microdissected from 85 cases, of which 30 (35%) showed LOH at some stage of progression. LOH was detected in the intraductal component in 26 of these 30 cases. Interstitial deletions were characterized by using a panel of ten highly polymorphic markers. The smallest region of overlap (SRO) for LOH at 11p15 was bounded by the markers D11S4046 and D11S1758. LOH at 11p15.5 showed no correlation with estrogen receptor status, the presence of positive lymph nodes, tumor size, histological grade, or long term survival. We conclude that 11p15 LOH usually occurs early in breast cancer development, but less frequently does not develop until the infiltrating or metastatic stages of tumor progression.

INTRODUCTION

Chromosome 11p15 shows high frequency loss of heterozygosity (LOH) in multiple human malignancies, including tumors of the breast,[1-7] lung,[8-12] cervix,[13] testis,[14, 15] bladder,[16] stomach,[17] and pediatric tumors of the adrenal and liver.[18, 19] This region has also been of interest because chromosomal breakpoints associated with the Beckwith-Wiedemann syndrome (BWS) [20] occur at this locus. In addition, physical transfer of chromosomal fragments into cell lines has provided functional evidence for one or more tumor suppressor genes in this region.[21, 22] The application of this technique to the breast cancer cell lines MDA-MB-435 and MCF7 has been shown to result in suppression of metastasis [23] and tumorigenicity, [24] respectively.

Previous studies of 11p15 LOH in breast cancer have revealed one smallest region of overlap (SRO) located in 11p15.5 between the markers TH and D11S988 (Fig. 1). [4, 6] These studies also provide evidence for additional SRO's in this region, one located more centromeric, in the 11p15.3 region [6] and the other more telomeric.[4] This locus may therefore contain several genes capable of contributing to the initiation or progression of breast cancer. Multiple SRO's in this region have also been reported in studies of lung carcinomas [8, 11] as well as in tumors of the adrenal and liver.[18]

In this study, LOH at 11p15 was characterized during breast cancer progression. Tumors were examined for the presence of intraductal, invasive, and metastatic foci. Each component present was isolated by microdissection and tested for LOH at a group of markers

that span the segment of 11p15 containing the reported SRO's for breast cancer. Our results indicate that LOH at 11p15 usually is present at the stage of intraductal carcinoma but occasionally does not occur until later stages of progression.

MATERIALS AND METHODS

Case selection. The material used in this study consisted of formalin fixed paraffin embedded tissue from the archives of the AFIP. The cases analyzed were selected from a group of 964 breast cancer cases submitted to the Armed Forces Institute of Pathology between 1975 and 1982 for which the patient's social security number was available to facilitate determination of vital status. A subset of these cases was selected for LOH analysis on the basis of the availability of normal tissue and the presence of intraductal lesions which were believed by the initial observers to be separable by microdissection from invasive and metastatic components of the tumor. Each tumor component present was isolated by microdissection from 12 μm sections which had been deparaffinized with Hemo-De (Fisher). Lysates were prepared from these tissue specimens by incubation in 200 μl of 10 mM Tris, pH 8.0/50 mM KCl/0.1 mM EDTA/0.5% Tween 20/100 μg/mL Proteinase K for 12-16 hr at 55°C followed by a 5 min incubation at 95°C to inactivate the protease. Insoluble material was pelleted by centrifugation for 5 min and the supernatant was used as the source of DNA template for PCR.

LOH Analysis. All tumor components were analyzed for LOH at the polymorphic markers D11S922, D11S988, and TH (Tyrosine Hydroxylase), which lie within or near the boundaries of a previously identified minimal region of overlap at 11p15.5,[4,6] and D11S837, which maps within a group of potentially growth regulatory genes at 11p15.3, a region that might represent a distinct SRO in breast cancer. [25-27] Six additional markers taken from the Genethon panel were used to characterize the SRO in cases with LOH. The order assigned

(Fig. 1) is based on the Genethon linkage data and physical mapping studies. [28-30] PCR reactions were performed in the presence of one ^{32}P-end labeled primer. Products were resolved on 6% polyacrylamide/7 M urea gels and visualized by autoradiography. The ratio of alleles present was initially evaluated by visual inspection of an appropriately exposed autoradiogram. When allele loss was partial, presumably due to the presence of normal cells in the microdissected specimens, band intensities were quantitated with a Molecular Dynamics Storm system, and reduction in allele ratio of greater than 50% was scored as LOH. Results were considered uninformative if the normal tissue was homozygous, if the tissue lysate failed to amplify, or if the results could not be interpreted unambiguously. Because nonamplifiable specimens were scored as uninformative, the percentage of cases reported as uninformative for each marker studied is greater than the percentage of homozygotes.

Statistical Analysis. Statistical analysis was performed using the Statistica program package (Release 5.1, StatSoft, Tulsa, OK). Correlations were assessed using contingency table analysis or regression methods, as appropriate. Survival analysis was performed using Kaplan-Meier plots.

RESULTS

Microdissection of tumor components. Tumor components used for DNA isolation were identified microscopically on deparaffinized but unstained sections of formalin fixed paraffin embedded tissue. An adjacent section stained with hematoxlyin and eosin was examined to confirm the histologic identification of the tumor components selected for analysis. Areas of intraductal, infiltrating, and metastatic tumor were dissected from the unstained sections under microscopic observation, and tissue lysates suitable for PCR analysis were prepared as described in Materials and Methods. A total of 115 cases were dissected in this manner. Of these, eight were intraductal carcinomas without evidence of invasion, 59 had progressed to the invasive stage but were node negative, and 48 had lymph node metastases. In addition, three cases (#16, 90, and 103) had carcinomas of the contralateral breast subsequent to the primary tumor, and two cases (#77 and 105) had distal metastases from which tissue was available for analysis. All of the tumor components were analyzed for LOH at the markers D11S922, TH, D11S988, and D11S837 in order to identify cases with 11p15 LOH and to establish the stage of progression at which LOH occurred. Specimens demonstrating LOH with one or more of these markers were further studied with the other six markers in our panel to characterize the SRO.

LOH at 11p15 during breast cancer progression. A total of 43 of the 115 cases (37%) analyzed demonstrated LOH in at least one tumor component. Samples of intraductal carcinoma were available for analysis in 30 of the 43 cases (70%) showing LOH at 11p15. Three of the eight pure intraductal carcinomas (37.5%) had LOH. LOH was detected in 19 of

the 59 (32%) node negative cases that had progressed to the invasive stage. Intraductal components could be identified and dissected from 15 of these 19 cases, and LOH was detected in 13 of the 15 intraductal specimens. Of the 48 cases with lymph node metastases, 21 (44%) demonstrated LOH in at least one tumor component. 12 cases in this group had dissectable intraductal components, with 10 showing LOH. 15 of 18 specimens of invasive carcinoma in this group, and 18 of the 21 metastases demonstrated LOH. The results are summarized in Table 1.

LOH at 11p15 was therefore most commonly observed in the intraductal component of these tumors and maintained throughout subsequent stages of progression. However, several exceptions were observed (Fig. 2). Of the cases with LOH, 27 had a dissectable intraductal component in addition to material representing later stages of progression. Of these cases, four (72, 78, 83, and 93) did not show LOH in the intraductal carcinoma. Similarly, there were three cases (78, 93, and 112) with LOH in the metastatic component but not in the corresponding specimen of invasive tumor. Interestingly, two cases (50 and 105) showed LOH in the intraductal and invasive specimens which was not detected in the lymph node metastasis. Of three cases with asynchronous contralateral tumors, two cases (16 and 90) demonstrated LOH in the primary tumor but not in the subsequent tumor in the opposite breast. In case 77, a pleural recurrance six years following excision of the primary tumor demonstrated LOH, whereas the primary tumor did not.

Inferences regarding the development of LOH during breast cancer progression could potentially be inaccurate because of contamination of the microdissected tumor components with normal cells, thereby obscuring the LOH present in certain tumor components. To rule

out this possibility, lysates from cases showing different patterns of LOH at different stages of progression were tested for LOH at various other loci. As shown in Fig. 2, the detection of LOH at loci on chromosome 17q in 2 of these cases (#50 and 72) demonstrates that the dissected material was of sufficient purity to detect LOH if it were present. Therefore, in a minority of cases, 11p15 LOH does not occur until progression beyond the intraductal stage.

Analysis of the SRO for LOH at 11p15. Cases showing 11p15 LOH were further analyzed with a panel of 10 loci spanning the region from 11p15.3 to 11p15.5 previously shown to contain SROs relevant to breast cancer (Figure 3). Of 43 cases with LOH, 19 showed LOH at all informative markers, while 24 provided evidence of interstitial deletions. Overall, the markers TH and D11S1318 appeared to lie within a major SRO, with each marker detecting LOH in 100% of informative cases, whereas markers flanking this region on either side showed reduced sensitivity for the detection of LOH. 20 of the 24 cases with interstitial deletions yielded results consistent with loss of a continuous segment of the chromosome. Data from four cases (41, 93, 101, and 103) suggest two regions of LOH, one centered on the markers TH and D11S1318, and the second located more proximally, at 11p15.3-15.4.

Examples of the LOH data from cases critical for establishing the SRO or for demonstrating two regions of LOH are presented in Fig. 4. Case 38 demonstrates LOH at the more telomeric of the markers analyzed, but shows retention of heterozygosity over the centromeric segment of this region. Case 93 is an example of a case with two regions of LOH separated by a region of retention of heterozygosity. Case 124 shows retention of heterozygosity at D11S4046, LOH at D11S1318, and retention of heterozygosity at more proximal markers.

Analysis of patterns of LOH in cases with interstitial deletions suggests a minimal region of overlap between D11S4046 and D11S1758. Two cases (#124 and 11) retained heterozygosity at D11S4046, indicating that the minimal region of overlap does not extend telomeric to this marker. At the centromeric border of this region, one case (#38) retained heterozygosity at D11S1758, and two (#38 and 101) at D11S4146, suggesting that these markers lie proximal to a minimal region of overlap.

Clinicopathological correlations. Survival curves for cases with and without LOH at 11p15 demonstrate no significant difference between the two groups (Figure 5). Other clinical and pathological data on these cases were reviewed. No relationship was found between LOH at this locus and estrogen receptor status, the presence of positive lymph nodes, S phase fraction, tumor size, histological grade.

DISCUSSION

Microdissection of histologic sections permits the isolation of tissue samples representing progressive stages in the evolution of breast cancer. We have applied this method to the analysis of LOH at chromosome 11p15, a locus known to show high frequency genetic alterations in breast cancer. Our results demonstrate several variant patterns for the occurance of LOH at this locus during breast cancer progression. Most commonly, we observed LOH in the earliest stage available for analysis. In four cases, however, LOH was absent from the intraductal tumor but present in later stages of progression. Interestingly, in two cases LOH seen in an invasive tumor was not detected in synchronous lymph node metastases. The latter observation indicates that invasive and metastatic components of an individual tumor can, at least in a small proportion of cases, represent genetically divergent clones rather than progressive stages in the clonal evolution of the tumor. Although the frequency with which genetically divergent clones arise during breast cancer evolution cannot be determined from the present study, the phenomenon has implications for the development of therapies targeted at specific growth regulatory signaling pathways, and therefore merits further investigation.

Our observation that 11p15 LOH is usually present by the time the tumor has progressed to the intraductal carcinoma stage is of interest with respect to the report that LOH can be observed in morphologically benign terminal duct lobular units (TDLUs) adjacent to foci of intraductal carcinoma.[31] In that study, only one of five cases with LOH at 11p15.5 was found to have LOH in an adjacent TDLU, whereas chromosome 3p markers showed LOH with a much higher frequency in such morphologically normal specimens. This observation,

together with our finding that LOH at this locus is usually present in the intraductal carcinoma, suggests that loss of function of the tumor suppressor gene at this locus may often be an event involved in the progression from morphologically benign epithelium to intraductal carcinoma.

Pathological data and long term clinical follow-up for as long as 25 years following the initial diagnosis was available for most of the cases analyzed in this study. This information was used to evaluate 11p15 LOH as a potential prognostic marker. We observed no significant correlation between 11p15 LOH and survival, tumor stage, grade, or presence of metastases. Presumably, the significant genetic alteration at this locus occurs before the cell has acquired the aberrant growth characteristics necessary for invasion and metastasis. Although some studies have reported associations between LOH at 11p15 and clinical parameters, one report has suggested that these correlations may actually reflect LOH at 11q, which is believed to contain a distinct tumor suppressor gene, rather than at 11p.[6] Other investigators have also observed a lack of correlation between 11p15 LOH and clinical parameters.[4] The reasons for the apparent discrepancy between studies that find no correlation between 11p15 LOH and the development of metastases (this study, Ref. 4 and 6) and a report that such a correlation exists (Ref. 5) are not clear, but may reflect differences in the specific markers used or in the population of breast cancer patients analyzed.

This report provides confirmatory evidence for the existence of an SRO distal to D11S988 at 11p15.5. In our study LOH was identified in 43 of 115 (37%) cases, of which 24 had interstitial deletions. The overall percentage showing LOH is comparable to the 35% previously reported by Winquist et al.[6] in breast cancer, who also noted that LOH at this locus is most often interstitial. Similar percentages of LOH have been reported in other tumors,

including lung (43%),[11] bladder (30%),[16] stomach (41%),[17] and testis (59%).[14] The boundaries of the SRO appeared to be similar when data from each stage of tumor progression was analyzed separately; that is, we did not observe a widening of the SRO when the data from only infiltrating or only metastatic tumor components were analyzed, as might have been expected if additional genes from this region were involved in later stages of tumor progression. The four cases in which LOH was not observed in the intraductal component but was detected at a more advanced stage of tumor progression also yielded evidence of a similar SRO. These results are consistent with the possibility that the same gene can be involved at different stages of tumor progression.

The SROs at 11p15 in breast cancer inferred from this and previous studies include a major region of LOH centered on or near the marker D11S1318 plus one or more secondary regions. LOH at these secondary regions seems to occur only in those tumors which demonstrate LOH of the major region. The results of Winqvist et al.[6] identified the markers TH and D11S988 as boundaries of one minimal region of overlap. A second SRO was identified more proximally, an observation similar to our finding of several cases with two distinct regions of LOH. Data from another study [4] supported two independent regions of LOH at 11p15.5, one between D11S1318 and D11S988, the other located distal to D11S1318. The major SRO defined by our data set may therefore encompass more than one gene important in the etiology of breast cancer. Although several studies have now reported cases with distinct regions of LOH at 11p15, no cases have been reported in which LOH involves the proximal (to D11S988) or distal (to D11S1318) regions without also involving the major SRO between D11S988 and D11S1318. It remains unclear whether this implies that genes in

the proximal and distal SRO's become important only after inactivation of a gene in the central region, or, perhaps, that the mechanism of genetic loss may occasionally permit retention of a chromosomal segment when flanking sequences on both sides are deleted.

Minimal regions of overlap for LOH at 11p15 have been described in several tumor types other than breast cancer. In non-small cell lung cancer (NSCLC), one study [11] reported evidence for a telomeric SRO distal to TH, centered on D11S922, and a second region proximal to D11S988, centered on D11S909. The latter marker is close to D11S837, the most proximal marker used in the present study. As observed in other studies of LOH at 11p15.5, no cases demonstrated LOH exclusively at the more proximal locus. These results suggest that the major SRO observed in breast cancer (D11S988-TH) may not play a role in NSCLC. However, studies of several other tumor types point to minimal regions of LOH that contain the chromosomal segment identified as the major SRO for breast cancer. Characterization of 100 carcinomas of the bladder suggested a minimal region of overlap between D11S922 and D11S569,[16] a region that includes the D11S988-TH chromosomal segment. A study of 13 hepatoblastomas suggested a significant region of LOH distal to the HBB locus.[19] In an analysis of 60 adenocarcinomas of the stomach,[17] most cases were found to have LOH of all informative markers, but two cases suggested a minimal region of overlap bordered by D11S1318 and D11S988.

The chromosomal segment containing the major breast cancer SRO has also been implicated as the locus of genes involved in cancer by other methodologies. Direct transfer of chromosomal fragments allowed the mapping of a tumor suppressor activity for the cell line

G401 to 11p15.5.[21, 22] Using subchromosomal transferable fragments, a growth inhibitory activity for the rhabdomyosarcoma cell line RD was mapped to a similar region.[32]

Studies of chromosomal translocations in several human malignancies have mapped breakpoints within the common region of LOH at 11p15, providing further evidence for the presence of one or more tumor suppressor genes in this region. A breakpoint in a rhabdoid tumor was localized approximately 60 kb centromeric to the TH gene.[33] In a myeloid leukemia, an 11p15 breakpoint was found to represent a fusion between the Nup98 gene on 11p15 and the homeobox gene HOXA9 on chromosome 7.[34] Studies of BWS translocations have led to the identification of at least 11 genes within a 320 kb segment containing the more telomeric cluster of five breakpoints.[35] The potassium channel gene KVLQT1, which spans all five BWS breakpoints localized within these 320 kb, is strongly implicated in BWS.[36] The gene encoding the cyclin dependent kinase inhibitor p57Kip2 maps within this region and may also play a role.[37] This segment, extending to a point approximately 100 kb centromeric to the insulin/TH/IGF2 gene cluster, lies within the major SRO observed in breast cancer.

The remaining three BWS breakpoints occur within a 1.2 megabase region of band 11p15.3, approximately four megabases centromeric to the cluster of five breakpoints. This region contains three genes potentially involved in growth regulation: rhombotin, WEE1, and ST5.[25-27] Another candidate tumor suppressor, H19, lies less than 200 kb telomeric to IGF2. This gene lies telomeric to the D11S988-TH segment, but maps within the boundaries of the more telomeric SRO identified by Negrini et al.,[4] as well as within the SRO defined by the present study. The recently described tsg101 gene, which was found to undergo aberrant splicing in several breast carcinomas, localizes to 11p15.1, a locus centromeric to the region

containing the BWS breakpoints and the common region of LOH identified in multiple tumor types.[38, 39]

In summary, characterization of LOH at 11p15 in a panel of breast carcinomas demonstrated that LOH usually occurs at this locus by the time the disease has developed to the intraductal carcinoma stage, but may occur at a later stage of progression. Analysis of the SRO at this locus supports localization of a tumor suppressor gene involved in breast cancer to a region similar to loci identified by chromosome transfer studies and analysis of BWS breakpoints, suggesting that the same gene or genes at 11p15 may be involved in BWS, growth suppression detected by chromosome transfer, and breast cancer.

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FIGURE LEGENDS

Figure 1. Location of markers used in this study and candidate tumor suppressor genes. The upper map shows the position of several genes, including the candidate tumor suppressor genes H19 and KIP2, along the region of approximately 6 Mb analyzed in this study. The lower map shows the positions of the genetic markers used. The map positions of the Genethon markers are from the sex averaged Genethon Human Linkage Map.[28] The distances between Genethon markers are indicated below the map in centiMorgans. The marker D11S837 (ST5 gene) has been localized within the centromeric group of BWS breakpoints.[40] The locations of the markers D11S988 and TH are from the CHLC/GDB map. The upper and lower maps are shown in approximate alignment. The KVLQT1 gene occupies approximately 300 kb and encompasses the more telomeric group of BWS breakpoints [36]. The TH gene is known to be closely linked to D11S1318, but the order of these two loci is not known with certainty. The H19 gene has been identified on a 100 kb bacterial artificial chromosome clone that also contains the marker D11S4046, suggesting close proximity of these loci in the genome.

Figure 2. LOH occurring at different stages in the progression of breast cancer. Alleles showing loss in tumor specimens are indicated by arrows. Allele ratios were determined by quantitating the bands on a Molecular Dynamics Storm system, calculating the ratio of (lost allele)/(retained allele), and dividing by the same ratio obtained with the normal tissue. Case 72: LOH first seen in invasive tumor at 11p15, but in intraductal at 17q; Case 112: LOH first detected in metastasis; Case 77: LOH not seen in primary tumor, but present in a pleural

metastasis six years later; Case 90: LOH present in primary tumor, but not in a contralateral tumor (labeled "recurrance") four years later; Case 50: LOH detected at 11p15 and 17q in invasive tumor but only at 17q in metastasis.

Figure 3. Sublocalization of the minimal region of LOH at 11p15 in breast cancer. Results obtained with the 43 cases showing LOH with at least one marker are presented. Solid circles: LOH; Shaded circles: Retention of heterozygosity; Open circles: Uninformative (homozygous or nonamplifiable); M: Microsatellite instability. The solid bar to the right of the figure indicates the minimal shared region of LOH inferred from this data set.

Figure 4. Patterns of LOH at 11p15 in breast cancer. A. Case 38: LOH at the distal group of markers, retention of heterozygosity at D11S1758, D11S4146, and D11S988, defining centromeric border of SRO. B. Case 93: LOH at proximal and distal markers with retention of heterozygosity between these two regions. C. Case 124: Retention of heterozygosity at D11S4046, defining telomeric border of SRO. N: normal tissue; T: tumor. Arrowheads indicate the allele lost in assays showing LOH. When LOH was present, quantitation was as in Figure 2. In cases where there was no LOH, the numerator and denominator of the reported allele ratio were chosen so as to yield a number less than the ideal value of 1.0.

Figure 5. Cumulative proportion surviving (Kaplan-Meier). Survival curves were generated for cases with (solid lines) and without (dashed lines) LOH at 11p15.

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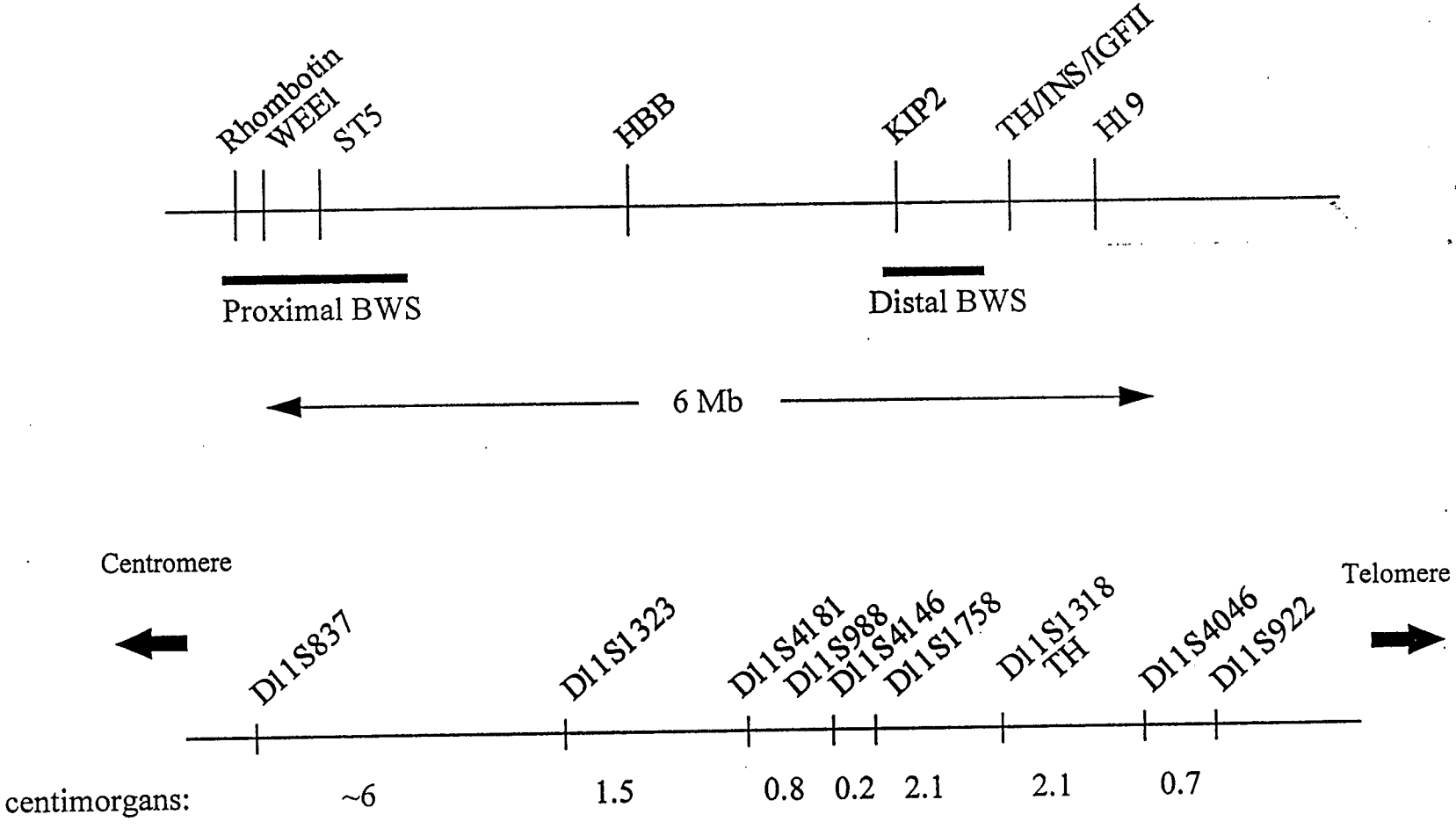
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WEE1
ST5
HBB
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TH/INS/IGFII
H19
Proximal BWS
Distal BWS
6 Mb
Centromere
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D11S837
D11S1323
D11S4181
D11S988
D11S4146
D11S1758
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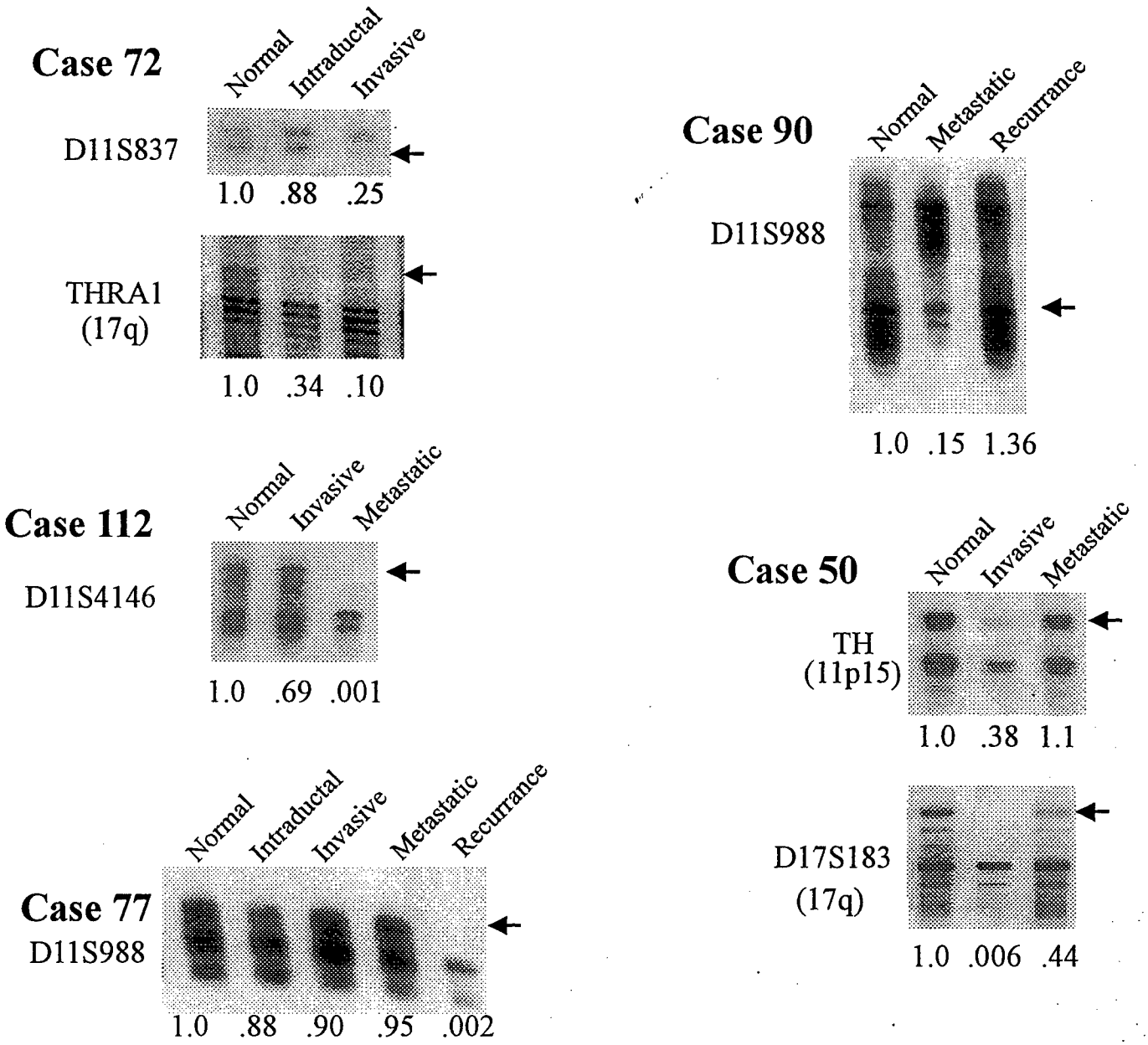
Case 72
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Intraductal
Invasive
D11S837
1.0 .88 .25
THRA1
(17q)
1.0 .34 .10
Case 90
Normal
Metastatic
Recurrance
D11S988
1.0 .15 1.36
Case 112
Normal
Invasive
Metastatic
D11S4146
1.0 .69 .001
Case 50
Normal
Invasive
Metastatic
TH
(11p15)
1.0 .38 1.1
D17S183
(17q)
1.0 .006 .44
Case 77
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Intraductal
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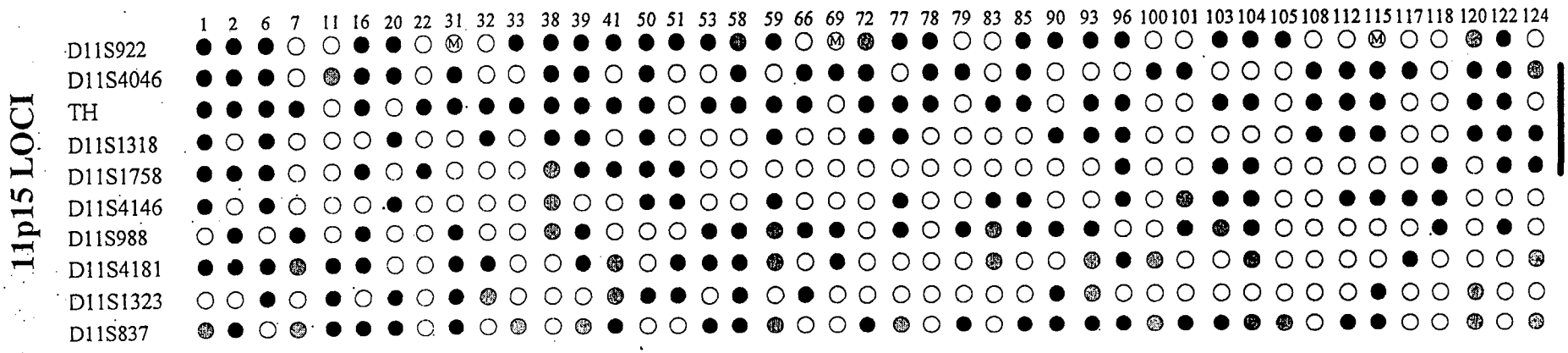
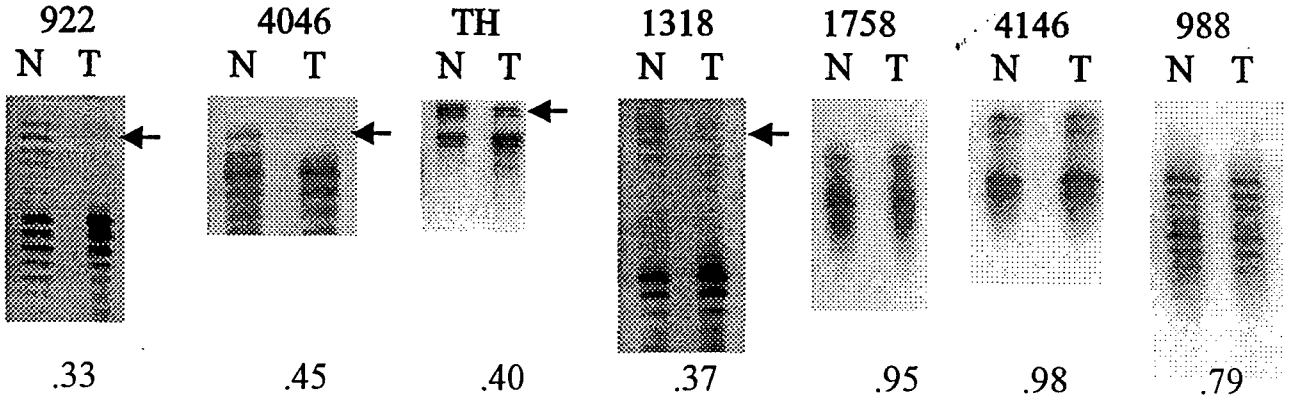
Metastatic
Recurrance
D11S988
1.0 .88 .90 .95 .002

BREAST CANCER CASE



A. Case 38



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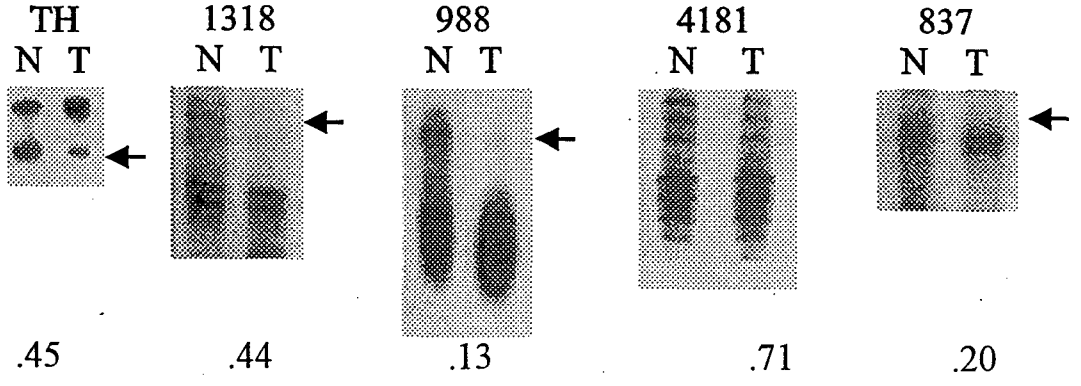

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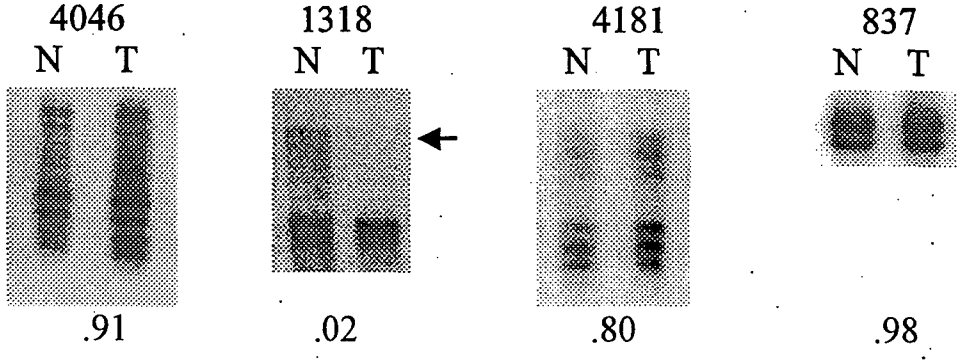

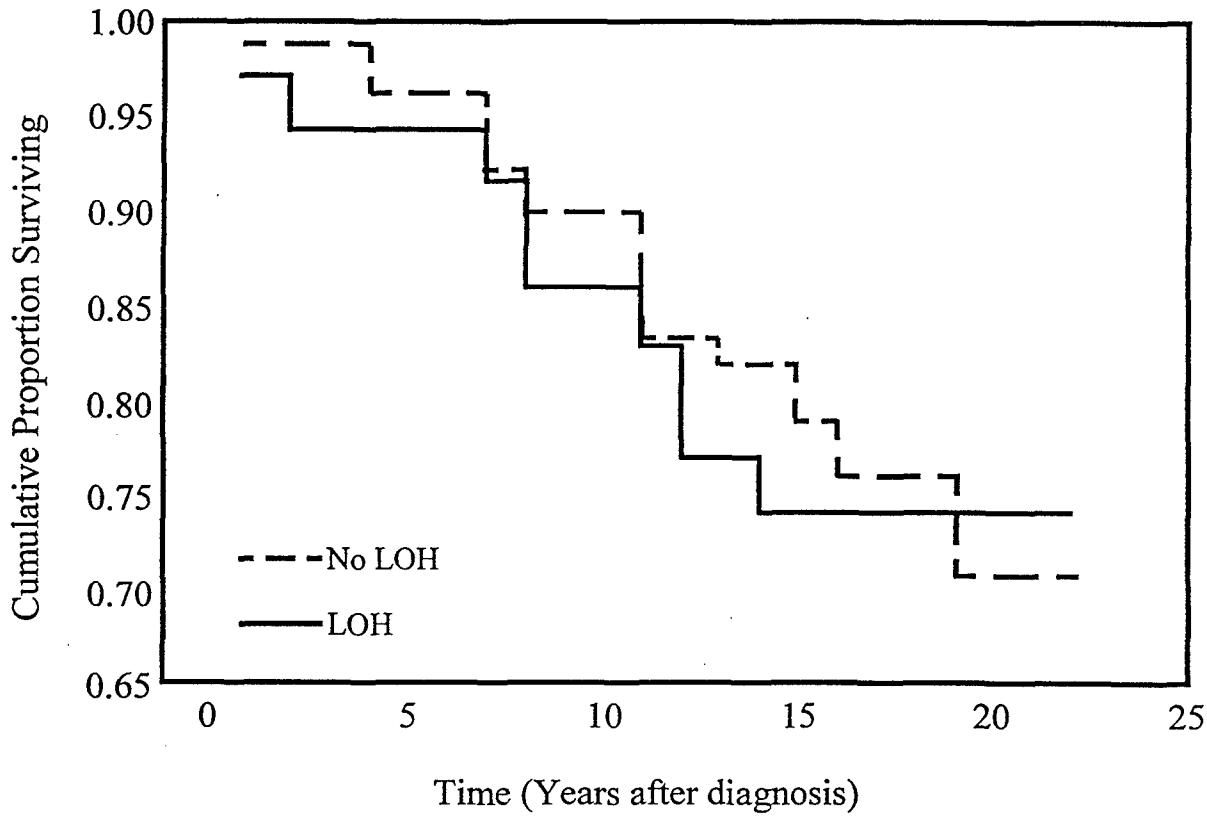
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
Cumulative Proportion Surviving
No LOH
LOH
0
5
10
15
20
25
Time (Years after diagnosis)

Table 1. Summary of LOH data during histologic progression.

Histologic Diagnosis:	No. Cases	LOH at 11p15 by stage of progression:			
		Any Component	**Intraductal**	**Invasive**	**Metastasic**
Intraductal carcinoma only	8	3 (38%)	3/3 (100%)	NA	NA
Invasive carcinoma without metastases	59	19 (32%)	13/15 (87%)	19/19 (100%)	NA
Invasive carcinoma with metastases	48	20 (42%)	10/12 (83%)	15/18 (83%)	18/20 (90%)